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Development and ultrastructure of *Besnoitia oryctofelisi* tachyzoites, tissue cysts, bradyzoites, schizonts and merozoites

J.P. Dubey[a,*], D.S. Lindsay[b]

[a]*United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, BARC-East, Beltsville, MD 20705-2350, USA*
[b]*Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061-0342, USA*

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Abstract

Development and structure of different life cycle stages of *Besnoitia oryctofelisi* which has a rabbit–cat life cycle was studied by light and transmission electron microscopy. For light microscopy, *Besnoitia oryctofelisi*-infected tissues were stained with haematoxylin–eosin, periodic acid Schiff (PAS) reagent, and immunohistochemically with rabbit anti-*B. oryctofelisi* polyclonal antibodies and anti-BAG-1 antibodies. In vitro and in vivo-derived tachyzoites were 5–6 μm long and they were found to divide by endodyogeny. In tachyzoites, the nucleus was often central, and micronemes were few and located anterior to the nucleus. Earliest tissue cysts were seen in gerbils starting 12 days p.i. Early tissue cysts had an outer PAS-positive cyst wall, a middle PAS-negative host cell layer, and an inner PAS-negative parasitophorous vacuolar membrane. Organisms in early tissue cysts were PAS-negative, did not stain with anti-BAG-1 antibodies, and amylopectin granules and enigmatic bodies were absent. Tissue cysts beginning 17 days p.i. contained organisms that became PAS-positive and reacted with anti-BAG-1 antibodies, indicating they were bradyzoites. Immunoreactivity with polyclonal anti-*B. oryctofelisi* antibodies suggested that *Besnoitia* species bradyzoites are encapsulated by the host cell. Bradyzoites (10 μm) were about twice the length of tachyzoites and contained enigmatic bodies characteristic of *Besnoitia* bradyzoites. Unlike tachyzoites and tissue cysts, schizonts were located intravascularly in the lamina propria of the small intestine of cats. Merozoites were 5–6 μm long, had few rhoptries and amylopectin granules, had numerous micronemes and had a terminal nucleus.

Keywords: Development; *Besnoitia oryctofelisi*; Ultrastructure; Schizont; Merozoite; Tachyzoite; Tissue cyst; Bradyzoite

1. Introduction

Besnoitia spp. are coccidians affecting livestock and wild animals (Frenkel, 1977; Leighton and Gajadhar, 2001; Paperna and Lainson, 2001; Dubey et al., 2003). Until recently, there were seven named species of *Besnoitia*: *Besnoitia bennetti* Babudieri, 1932; *Besnoitia besnoiti* (Marotel, 1912) Henry, 1913; *Besnoitia caprae* Njenga, Bwangamoi, Mutiga, Kangethe and Mugera, 1993; *Besnoitia darlingi* (Brumpt, 1913) Mandour, 1965; *Besnoitia jellisoni* Frenkel, 1953; *Besnoitia wallacei* (Tadros and Laarman, 1976) Frenkel, 1977, and *Besnoitia tarandi* (Hadwen, 1922), Levine, 1961. We recently described a new species of *Besnoitia, Besnoitia oryctofelisi*, with a rabbit–cat life cycle (Dubey et al., 2003). In the present paper we report the development and ultrastructure of *B. oryctofelisi* tachyzoites, tissue cysts, bradyzoites, schizonts, and merozoites.

2. Materials and methods

2.1. Infection of animals

In several experiments, gerbils and interferon gamma gene knockout (KO) mice were fed graded doses of sporulated *B. oryctofelisi* oocysts as described (Dubey et al., 2003). Animals that died or those euthanised were necropsied. For the study of tissue cyst development, the gerbils were examined 4, 7, 8, 12, 13, 16, 17, 25, and 49 days p.i. and the KO mice were examined 8, 10, 13, 14, 16 and 17 days p.i.

* Corresponding author. Tel.: +1-301-504-8128; fax: +1-301-504-9222.
E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

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Specimens of brain, heart, eyes, lungs, liver, spleen, tongue, kidneys, adrenals, and uterus, urinary bladder and leg muscles were fixed in 10% neutral buffered formalin. Paraffin-embedded sections were cut at 5 μm thickness and examined after staining with haematoxylin–eosin (H&E) or Gomori's reticulum stain (Manual of Histologic Methods, third edition, Armed Forces Institute of Pathology, Washington, DC, page 87), or periodic acid Schiff (PAS) reaction counter-stained with haematoxylin (PASH).

2.2. Cell culture

Tachyzoites of *B. oryctofelisi* were grown in CV-1 cells as described (Dubey et al., 2003).

2.3. Transmission electron microscopy

For transmission electron microscopy (TEM), tissues were fixed in Karnovsky fixative (Karnovsky, 1965) or in 10% buffered neutral formalin, post-fixed in osmium, and processed for TEM of tissue cysts. Liver from a KO mouse 17 days p.i., and muscles from rabbit No. 1, 54 days p.i. (Dubey et al., 2003), were used to study tissue cysts. For the study of tachyzoites, in vitro infected CV-1 cells, and mesenteric lymph nodes of a KO mouse fed oocysts 8 days earlier were used. Schizonts were from the jejunum of cat No. 687, 11 days after it was fed tissue cysts (Dubey et al., 2003).

2.4. Immunohistochemical staining

For immunohistochemical staining, two types of antibodies, and a Dako Envision Peroxidase (Dako, Carpinteria, CA) rabbit kit and Envision system were employed. The polyclonal anti-*B. oryctofelisi* antibody was from rabbit No. 1, bled 54 days after inoculation with tachyzoites (Dubey et al., 2003). This polyclonal *B. oryctofelisi* antibody was used at a 1:40,000 dilution to locate parasites in tissue sections. The bradyzoite-specific (BAG-1, also called BAG-5) rabbit antibody directed against a heat-shock protein from *Toxoplasma gondii* was supplied by McAllister et al. (1996). It was used in a 1:10,000 dilution; this antibody does not stain tachyzoites but does stain bradyzoites of *T. gondii* and *Neospora caninum* (McAllister et al., 1996). This BAG-1 antibody was used to trace the development of bradyzoites in tissue sections. For the Envision system, the procedure recommended by the manufacturer was followed. Briefly, after deparaffinisation, sections were treated with quenching solutions (3% hydrogen peroxide in absolute methanol), rinsed with water, digested in 0.4% pepsin, rinsed in blocking solution (0.5% casein), treated with primary antibody at 37 °C for 30 min, rinsed, and treated for 30 min with Dako Envision kit labelled Polymer HRP Solution, rinsed with buffer, treated with substrate 3 amino-9-ethylcarbazole, rinsed with buffer, counterstained with Mayer's haematoxylin, covered with Crystal Mount, dried at 37 °C overnight, and mounted in Permount.

3. Results

3.1. Tachyzoites

In tissues of gerbils and mice killed 4–10 days p.i. and in cell cultures, only tachyzoites were seen (Figs. 1 and 2). They were enclosed in a thin parasitophorous vacuolar membrane (PVM) without any hypertrophy of the host cell nucleus. Tachyzoites divided by endodyogeny (Figs. 1 and 2). Distinctive features of tachyzoites were a subpellicular thickening at the conoidal end and no amylopectin granules (Table 1). In tachyzoites, the nucleus was often central in location, and micronemes were few and often arranged in rows. The structure of in vitro-derived tachyzoites was essentially the same as in vivo-infected cells except that dense granules were more numerous within in vivo-derived than in vitro-derived tachyzoites (Figs. 1 and 2). Tachyzoites in vivo were seen in mononuclear cells and in neutrophils (Fig. 2).

Table 1
Ultrastructural differences among merozoites, tachyzoites, and bradyzoites of *Besnoitia oryctofelis*

Characteristic	Tachyzoites	Bradyzoites	Merozoites
Size (μm)	5–6 × 2.0[c]	9–10 × 1–2[d]	4.9–5.7 × 1.3–1.5[e]
Subpellicular thickening[a]	Present	Absent	Absent
Micronemes[b]	Few (<50)	Numerous (>100)	Numerous (>200)
Rhoptries[b]	Up to 6, short	2, as long bradyzoite	2, short
Nucleus	Central to posterior	Central to posterior	Terminal
Enigmatic bodies	Absent	Present	Absent
Dense granules	Up to 25	<6	<6
Amylopectin granules	None	Numerous	<6

[a] At the conoidal end.
[b] Per section.
[c] In smears (Dubey et al., 2003).
[d] In sections (Dubey et al., 2003).
[e] Present study.

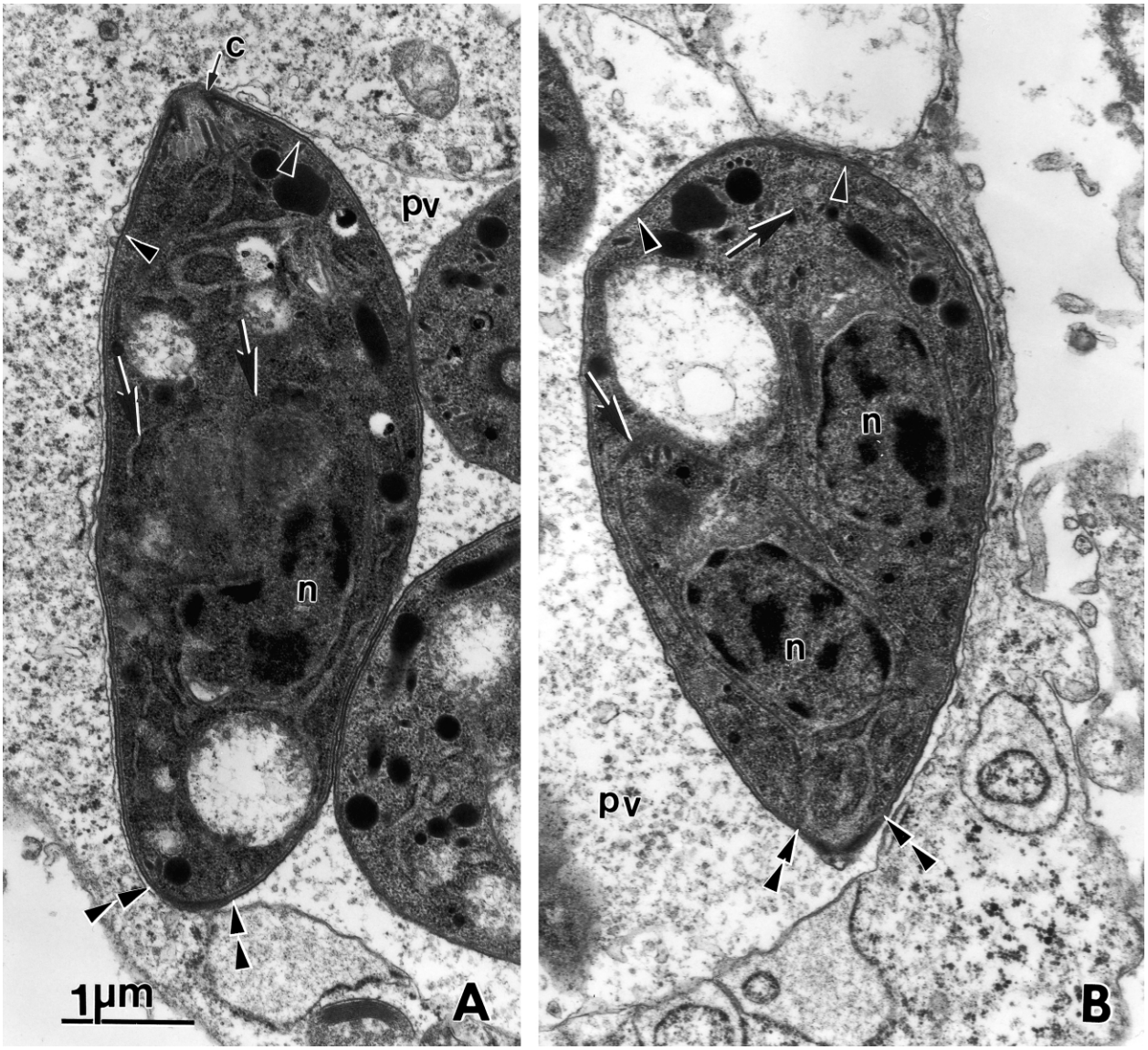

Fig. 1. Two *Besnoitia oryctofelisi* tachyzoites dividing by endodyogeny in cell culture. Conoidal ends of tachyzoites are facing up and have a subpellicular electron-dense thickening (arrowheads). Their non-conoidal ends have a polar thickening (double arrowheads). Organisms are enclosed in parasitophorous vacuoles (pv) in host cell cytoplasm. Bar applies to both parts. (A) Two developing tachyzoites. Note conoidal ends (arrows) of daughter zoites, the bilobed parent nucleus (n), and the parental conoid (c). (B) Two daughter tachyzoites (arrows) with their own nucleus (n).

3.2. Tissue cysts and bradyzoites

Earliest tissue cyst-like structures were seen in gerbils 12 days p.i. Host cells with hypertrophied nucleus or nuclei were seen to contain one or more organisms (Fig. 3A–C). Organisms in these 'young tissue cysts' had a central nucleus, were PAS-negative, and did not stain with anti-BAG-1 antibody. Up to five host cell nuclei were seen in the tissue cyst wall (Fig. 3C). The cyst wall in some of these young tissue cysts was PAS-positive (Fig. 3B,C) and argyrophilic. Beginning at 17 days p.i., some of the organisms within the tissue cysts developed PAS-positivity and some reacted with anti-BAG-1 antibody (Fig. 4C).

Beginning at 25 days p.i., all cystic organisms were PAS-positive and stained with BAG-1 antibody (Figs. 3C and 4D). Tachyzoites, PVM, and the cystic organisms reacted with anti-*B. oryctofelisi* polyclonal antibody whereas only bradyzoites reacted with anti-BAG-1 antibody (Fig. 4A–D).

Well-developed tissue cysts had three layers: an outer, a middle, and an inner layer. The outer layer (No. 1) was PAS-positive, and argyrophlic (Figs. 3 and 4). The middle layer (No. 2) enclosed host cell elements including host cell nuclei, and was PAS-negative. The innermost layer (layer No. 3) was PAS-negative and enclosed bradyzoites.

Ultrastructurally, the youngest tissue cysts contained as few as one zoite; these organisms were enclosed in an ill defined PVM. The PVM was surrounded by the electron-dense host cytoplasm (layer No. 2) with enormous endoplasmic reticula (Fig. 5). Layer No. 2 was surrounded by a homogenous acellular layer (layer No. 1). Zoites in

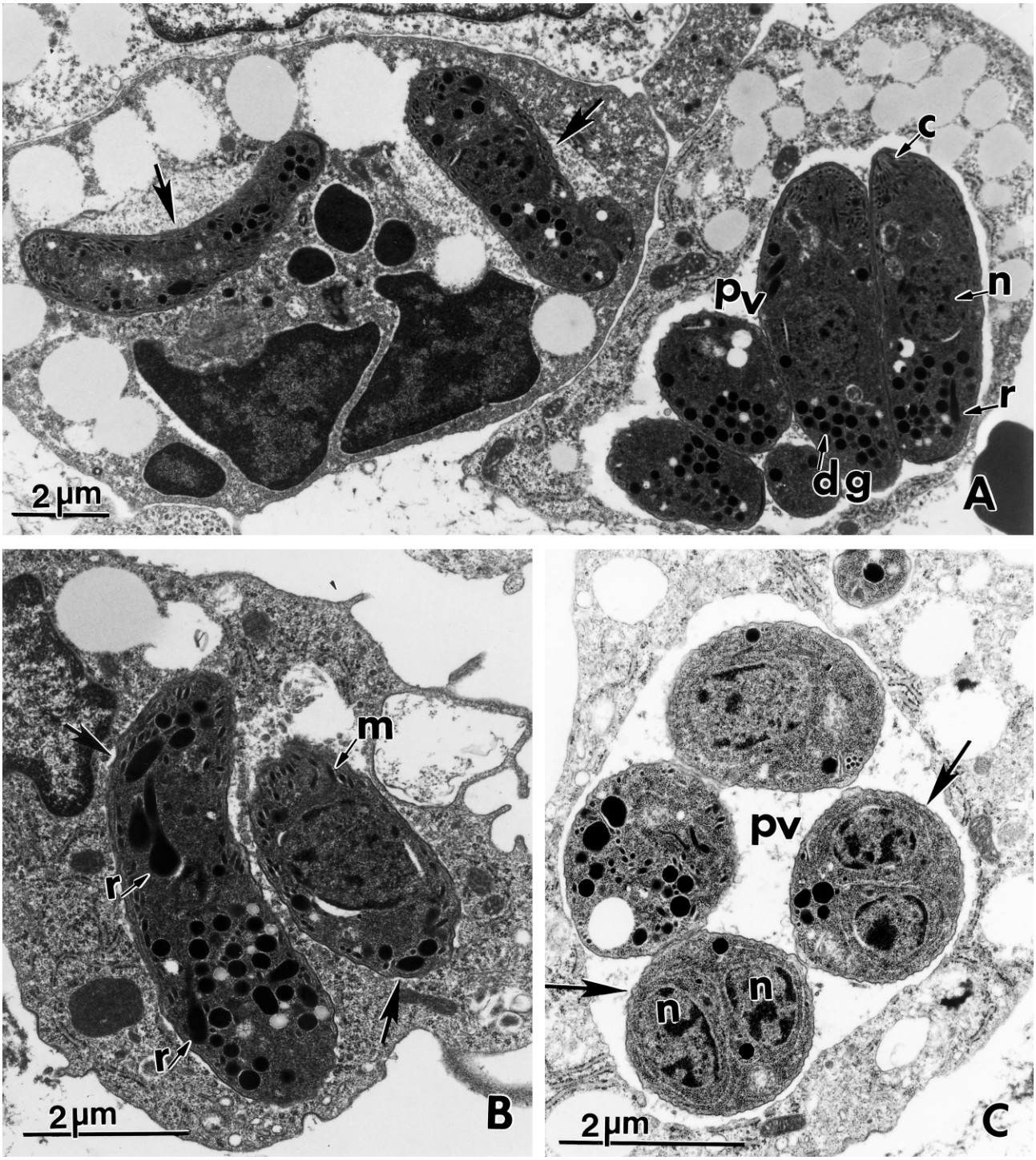

Fig. 2. Transmission electron microscopy of intracellular tachyzoites in mesenteric lymph node of a knockout mouse, 8 days p.i. Note numerous dense granules (dg), a conoid (c), rhoptries (r) extending posterior to the nucleus, and numerous micronemes (m) in tachyzoites. (A) Two individual tachyzoites (arrows) in a neutrophil on the left and five tachyzoites in a parasitophorous vacuole (pv) in macrophage cytoplasm on the right. (B) Two tachyzoites presumably in separate vacuoles (arrows). (C) Four organisms in a pv. Two organisms (arrows), each with paired daughter zoites.

these young tissue cysts contained a few micronemes, rhoptries and a central nucleus, but lacked amylopectin granules and enigmatic bodies (Figs. 6 and 7). In older tissue cysts, layer No. 2 was compressed against layer No. 1 (Fig. 8). Layer No. 2 had projections embedded into layer No. 1 (Fig. 8).

Numerous bradyzoites were located in amorphous material lining the PVM, which continued into the interior of the cyst between bradyzoites. Bradyzoites were distinguished from tachyzoites and merozoites by their longer size, numerous amylopectin granules, and characteristic enigmatic bodies (Table 1). Enigmatic bodies had an

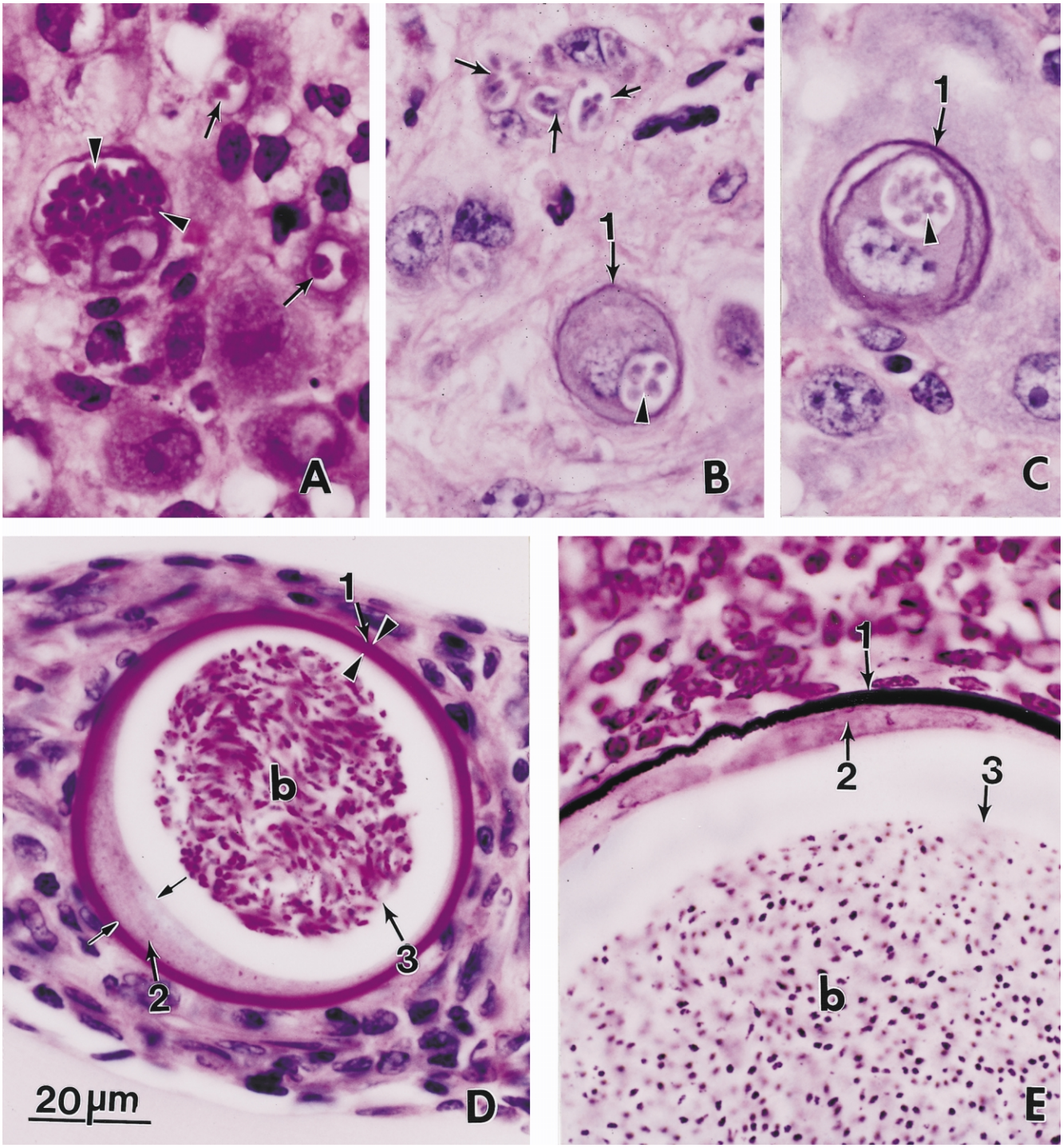

Fig. 3. *Besnoitia oryctofelisi* stages in sections of tissues of gerbils or mice fed oocysts. (A) Early tissue cyst-like structures in the lamina propria of small intestine of a gerbil, 12 days p.i. Haematoxylin–eosin. Two vacuoles each with two tachyzoites (arrows) and a binucleated host cell with a group of tachyzoites (arrowheads). (B,C) Tachyzoites (arrows) and early tissue cysts, liver of a knockout mouse, 17 days p.i. The outer tissue cyst wall (layer No. 1) is stained positively with periodic acid Schiff (PAS), and the enclosed organisms are PAS-negative organisms (arrowheads). PAS–haematoxylin. (D) A well-developed tissue cyst in the heart of a gerbil, 49 days p.i. This tissue cyst is surrounded by inflammatory cells. The tissue cyst has an outer layer No. 1 which is deeply stained with PAS (opposing arrowheads), a middle layer No. 2 (opposing arrows) and the innermost layer No. 3 that are both PAS-negative. All bradyzoites are stained red with PAS. PAS–haematoxylin. (E) A well-developed tissue cyst in mesenteric lymph node of a gerbil, 32 days p.i. Note only layer No. 1 and bradyzoite nuclei are stained whereas tissue cyst layers Nos. 2 and 3 are unstained. Gomori's silver stain. The empty space in (D,E) is probably a fixation artefact.

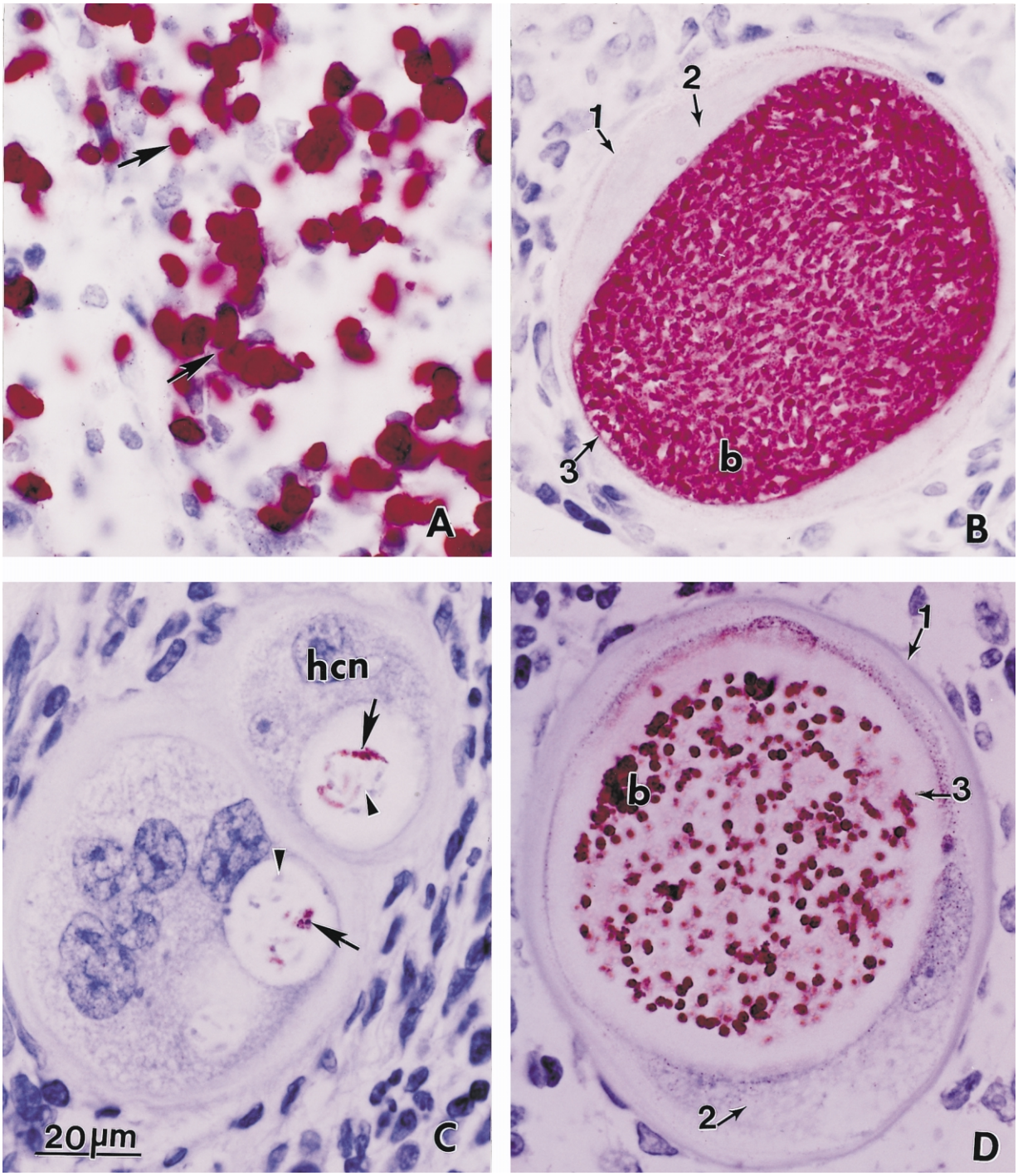

Fig. 4. Tachyzoites and tissue cysts of *Besnoitia oryctofelisi* in sections stained immunohistochemically with polyclonal rabbit *B. oryctofelisi* antibody (A,B) and with anti-BAG-1 antibody (C,D). (A) Numerous tachyzoites (arrows) in mesenteric lymph node of gerbil, 7 days p.i. (B) A tissue cyst in the lung of a gerbil, 49 days p.i. The innermost layer No. 3 (pvm) and enclosed bradyzoites (b) are stained with *B. oryctofelisi* antibody whereas the outer two tissue cyst wall layers (Nos. 1, 2) are unstained. (C) Two young tissue cysts in the liver of a knockout mouse, 17 days p.i. Note enlarged host cell nuclei (hcn). Some of the organisms are stained with anti-BAG-1 antibody (arrows) whereas others are unstained (arrowheads). (D) A well-developed tissue cyst. The bradyzoites are stained with anti-BAG-1 antibody whereas the tissue cyst layers (Nos. 1–3) are unstained. The structures not stained with antibody are stained with haematoxylin.

electron-dense core surrounded by an 'empty space' (Fig. 8). They were 295–475 × 90–135 nm in size and were located mostly towards the non-conoidal end (Fig. 8B). Up to 15 enigmatic bodies were seen in a given section of bradyzoite.

3.3. Schizonts and merozoites

Schizonts developed intravascularly, presumably in endothelial cells of the lamina propria of small intestine. The capillary lumen was often occluded by the schizont;

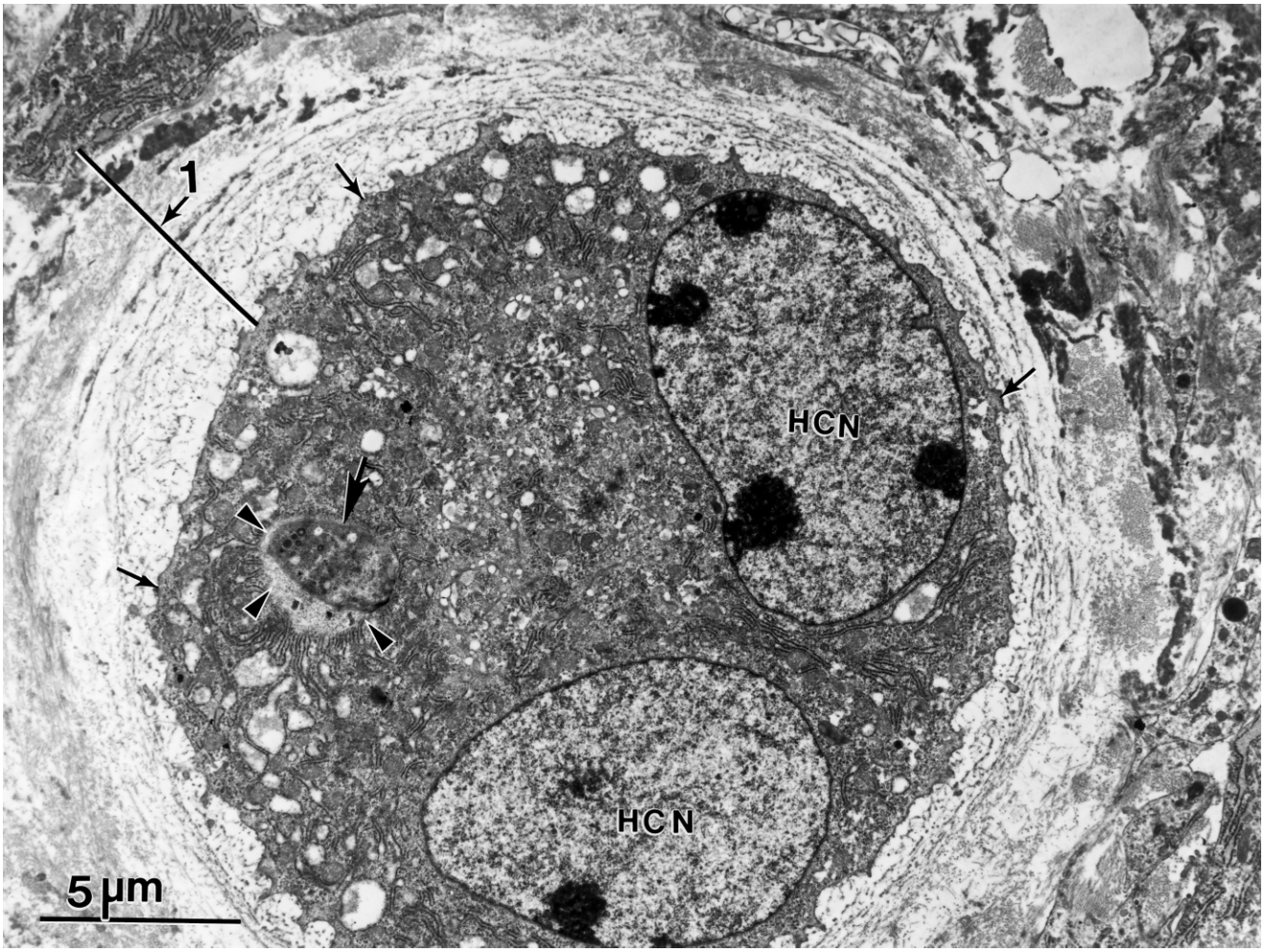

Fig. 5. Transmission electron micrograph of a young tissue cyst of *Besnoitia oryctofelisi* in the liver of a knockout mouse, 17 days p.i. Note three layers of the tissue cyst wall. Layer No. 1 consists of connective tissue like material, followed by layer No. 2 enclosing the host cell elements. The host cell is electron-dense and its boundary is demarcated by small arrows. The host cell layer encloses host cell nuclei (hcn) and the host cell cytoplasm has profuse endoplasmic reticula. Layer No. 3 is indistinct and encloses a small parasitophorous vacuole (demarcated by arrowheads) containing one zoite (arrow).

therefore blood cells were rarely visible around it. The host cell nucleus was hypertrophied even in schizonts with one or few nuclei. The parasite nucleus divided into numerous nuclei before merozoites were formed. As many as 100 merozoites were present in a given section of a schizont.

Four mature and one immature schizonts were studied ultrastructurally. Mature schizonts contained numerous merozoites enclosed in a PVM, adjacent to the host cell nucleus. Merozoites were often arranged in groups; the conoidal ends of most merozoites within a group were facing each other (Figs. 9 and 10). Merozoites were banana-shaped with pointed anterior (conoidal) end and a round posterior (basal) end; the nucleus was located towards the basal end. Ten longitudinally cut merozoites from four schizonts were 4.9–5.7 × 1.3–1.5 µm; the maximum width of other merozoites cut in cross-section was 2.0 µm. Distinctive features of merozoites were enormous numbers of micronemes arranged anterior to the nucleus and without any definite pattern (Fig. 10). Rhoptries were few and no more than two were seen in one plane of section of any merozoite (Fig. 10). Other organelles were a few amylopectin granules, a few dense granules, a mitochondrion, and a vacuolated area. Tubular structures and degenerated materials were dispensed among merozoites in the parasitophorous vacuole (Figs. 9 and 10).

4. Discussion

4.1. Tachyzoites

Most studies on the ultrastructure of tachyzoites are from tachyzoites of *B. jellisoni* (Sheffield, 1966; Sénaud et al., 1974; Sénaud and Mehlhorn, 1978). Sheffield (1966) remarked that the ultrastructure of *B. jellisoni* tachyzoites is remarkably similar to that of *T. gondii* tachyzoites. Paperna and Lainson (2001) described ultrastructure of in vivo-derived tachyzoites of a species of *Besnoitia* from the lizard *Ameiva ameiva* from Brazil and reported subpellicular electron-dense thickening toward the conoidal

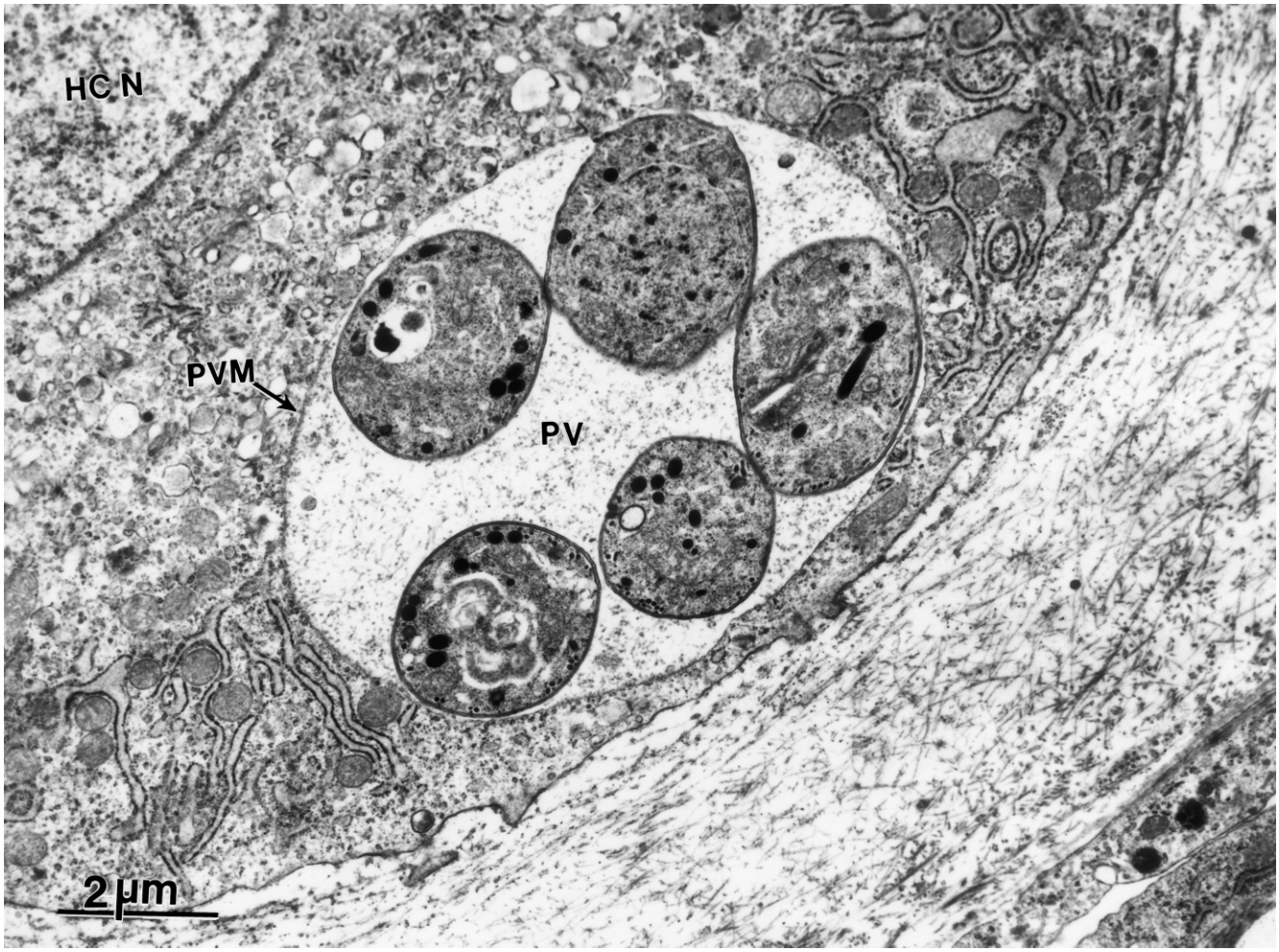

Fig. 6. Transmission electron micrograph of an early tissue cyst of *Besnoitia oryctofelisi* in the liver of the same mouse as in Fig. 2. Note five zoites in the parasitophorous vacuole (pv) enclosed in a thin parasitophorous vacuolar membrane (pvm). Electron-dense host cell elements surround the pvm. The zoites contain only a few micronemes, but no amylopectin granules or enigmatic bodies.

end; this finding was confirmed by Dubey et al. (2002) using cell culture-derived tachyzoites of *B. darlingi* and in the present study using in vivo and in vitro-derived tachyzoites of *B. oryctofelisi*. To our knowledge, this electron-dense thickening has not been found in tachyzoites of *B. jellisoni* or other *Besnoitia* species (Sheffield, 1966; Göbel et al., 1985; Shkap et al., 1988), or in *T. gondii*. Ultrastructure and measurements of tachyzoites of other species of *Besnoitia* are largely unknown (Dubey et al., 2003).

4.2. Tissue cysts and bradyzoites

Tissue cysts of *Besnoitia* are structurally distinct from tissue cysts or meronts of other coccidians because of the thickness of the tissue cyst wall and incorporation of host cell within the cyst. There is a confusion concerning the terminology used to define the structure of *Besnoitia* tissue cysts. Tissue cysts of *B. oryctofelisi* and all other species of *Besnoitia* consist of three layers; an outer, a middle, and an inner layer. The outermost layer is the same as the secondary cyst wall of Mehlhorn et al. (1974) and Heydorn et al. (1984), the cyst wall of Sheffield (1968), and external fibrous layer of Paperna and Lainson (2001). It consists of variable amounts of concentric to longitudinal filaments without any recognizable host cell organelles. It is PAS-positive (Sheffield, 1968; present study). The middle layer encloses host cell elements including nuclei; this layer is PAS-negative. The host cell nuclei are hypertrophied. The innermost layer (the PVM) is a thin membrane without any invaginations or protrusions, and surrounds bradyzoites. Because the thickness or the structure of the middle and outer layers of the *Besnoitia* tissue cysts may vary with duration of infection, types of cells and host parasitised, it is not of taxonomic value. For example, Paperna and Lainson (2001) found that the structure of the same *Besnoitia* species in the experimental host (mouse) was different from tissue cysts in the natural host (lizard).

The period to cyst development may vary with the route of inoculation, the species of *Besnoitia*, parasite stage inoculated, and the host (Sheffield, 1968; Basson et al., 1970; Paperna and Lainson, 2001; Dubey et al., 2003). In the present study, gerbils and mice were fed oocysts

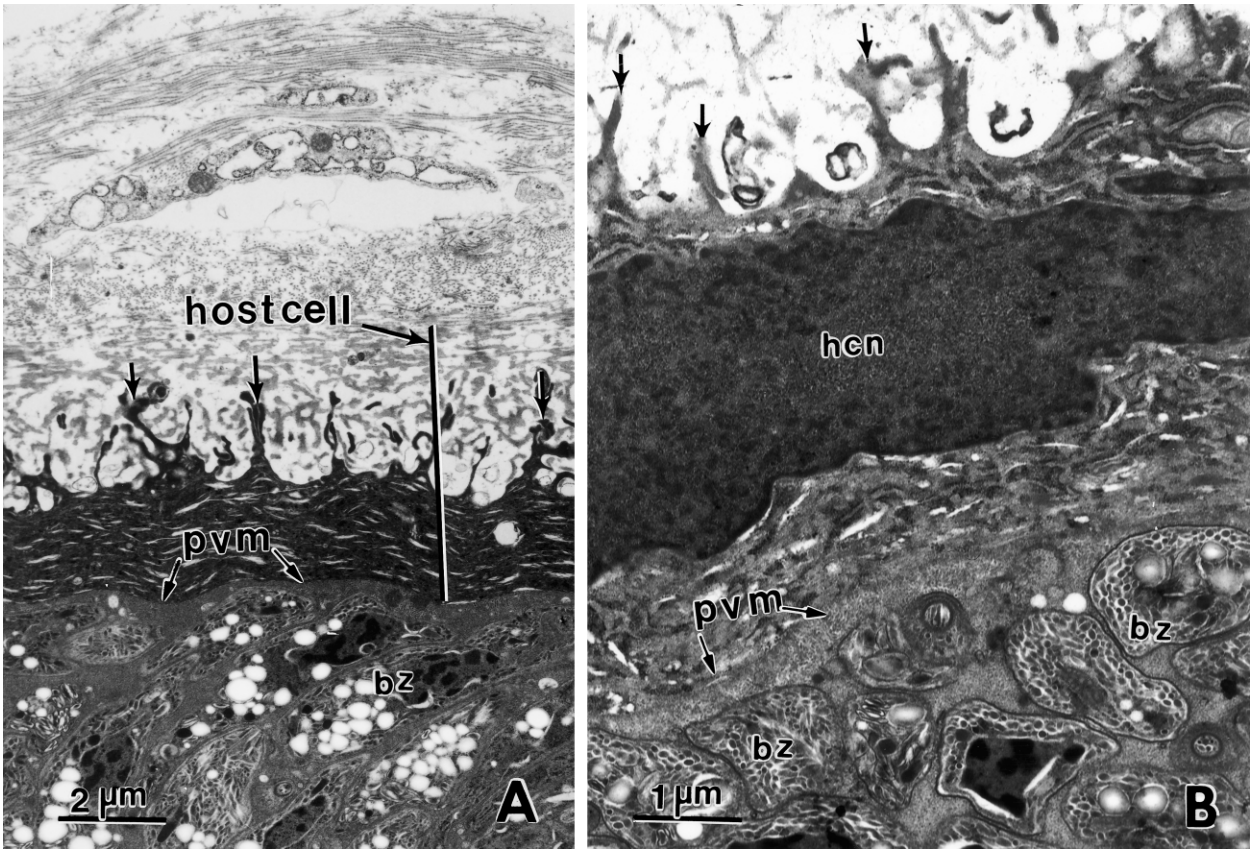

Fig. 7. Tissue cyst wall of *Besnoitia oryctofelisi* from muscle of an experimentally infected rabbit, 54 days p.i. Note several layers of host cell components on the outer layer of the parasitophorous vacuolar membrane (pvm). (A) An electron-dense layer with protrusions (arrows) of host cell components surrounds the pvm (arrowheads). (B) A host cell nucleus (hcn) is incorporated in the electron-dense layer outside the pvm. Note bradyzoites (bz) are densely packed and butted against the pvm.

whereas in previous studies on the tissue cyst development, animals were inoculated parenterally with tachyzoites. *B. jellisoni* tissue cyst formation was reported by Frenkel (1953, 1965), Sheffield (1968), Sénaud (1969) and Sénaud et al. (1974) after intraperitoneal inoculation of mice with tachyzoites. In *B. jellisoni*, earliest tissue cysts were seen 10–12 days p.i. (Frenkel, 1965), and Sheffield (1968) reported a tissue cyst wall around 4–8 organisms, 3–8 weeks p.i. Frenkel (1965) proposed that the cyst wall nuclei may be of parasitic origin because the cyst-wall formation can be abolished by rapid intraperitoneal passage of *B. jellisoni* in mice (Frenkel, 1965). Unlike *B. jellisoni*, which forms tissue cysts on the surface of viscera, *B. oryctofelisi* tissue cysts were found in parenchyma, predominantly in lungs, hearts, and adrenal glands (Dubey et al., 2003).

The outer *Besnoitia* tissue cyst wall layer and enclosed bradyzoites were PAS-positive. However, the biochemical nature of the cyst wall and bradyzoites of *B. jellisoni* are different; the cyst wall does not contain glycogen, whereas bradyzoites do (Sheffield, 1968). In *B. oryctofelisi* the outer tissue cyst wall and bradyzoites were PAS-positive whereas the cyst wall nuclei and the host cell cytoplasm were PAS-negative. In the present study, the cyst wall in young tissue cysts was PAS-positive whereas the enclosed organisms were either PAS-negative or contained only faintly stained PAS-positive granules. In the past, differential PAS-staining of these three layers of *Besnoitia* tissue cysts was not reported.

In the present study, organisms in young tissue cysts were first seen to be BAG-1 positive 17 days p.i., and not all organisms within a group were BAG-1 positive, suggesting that the gaining of a positive reaction to BAG-1 antibodies occurs after the tissue cyst wall has already formed. Results of PAS-positivity also support this hypothesis. There was a good correlation between PAS-positivity and positive reactivity to BAG-1 antibody suggesting that either method should be useful in studying tissue cyst formation. The organisms in young tissue cysts up to 17 days p.i. were considered transitional tachyzoites because they lacked amylopectin granules and enigmatic bodies characteristic of bradyzoites.

There is considerable debate regarding the origin of the cyst wall in *Besnoitia*. In the present study, only the PVM and enclosed organisms stained with anti-*Besnoitia* antibody and only bradyzoites stained with BAG-1 antibody. These data suggest that the parasite and the PVM are encapsulated by the host cell. Sheffield (1968) found that the

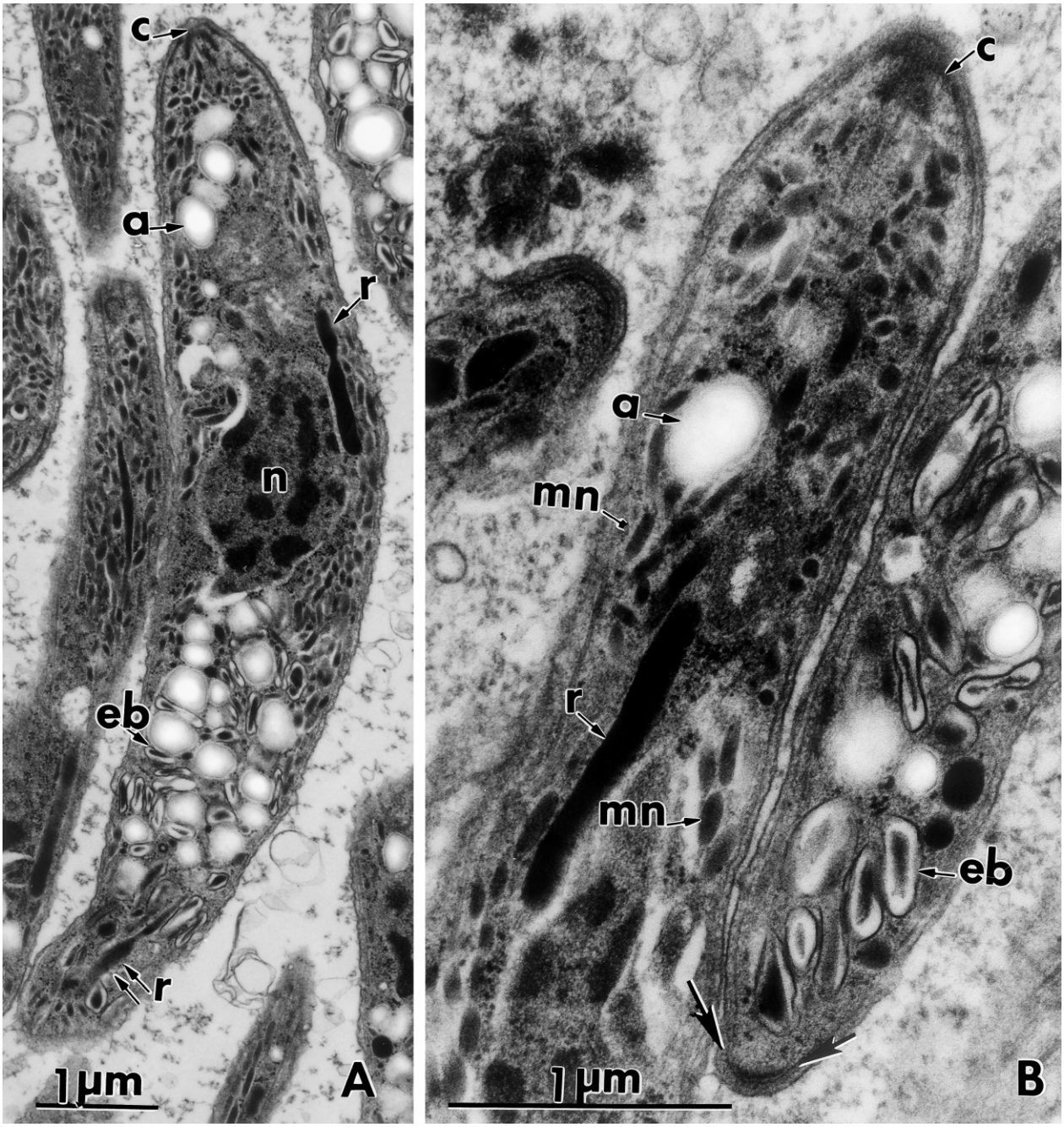

Fig. 8. Bradyzoites of *Besnoitia oryctofelisi*. Note conoid (c), amylopectin granules (a), nucleus (n), rhoptries (r), and enigmatic bodies (eb). (A) Longitudinal section with a conoid, a central nucleus, and a rhoptry (r) extending to the posterior end (double arrow). (B) Conoidal (c) and non-conoidal ends of two bradyzoites. Note enigmatic bodies (eb) are concentrated towards the non-conoidal end. Also note thickening (double arrows) of the non-conoidal end of one bradyzoite.

mitochondria in the cystic organisms (bradyzoites) differed structurally from mitochondria in the cyst wall, suggesting that the host contributed to the tissue cyst wall. Results of the present study using immunohistochemistry support this hypothesis because the cyst wall nuclei and the cyst-wall did not stain with anti-*Besnoitia* antibody.

Besnoitia tissue cysts are characterised by hypertrophy of the host cell nucleus and its inclusion in the cyst wall. Hypertrophy and division of the host cell nucleus is probably induced by tachyzoites destined to become bradyzoites because tachyzoites apparently do not induce hypertrophy of the host cell nucleus (Sénaud et al., 1974; Paperna and Lainson, 2001; Dubey et al., 2002).

At present there are no valid differences among bradyzoites of *B. jellisoni* (Sheffield, 1968; Sénaud, 1969; Sénaud et al., 1972, 1974; Scholtyseck et al., 1973; Mehlhorn et al., 1974), *B. tarandi* (Hilali et al., 1990), *B. darlingi* (Dubey et al., 2002), *B. caprae* (Heydorn et al.,

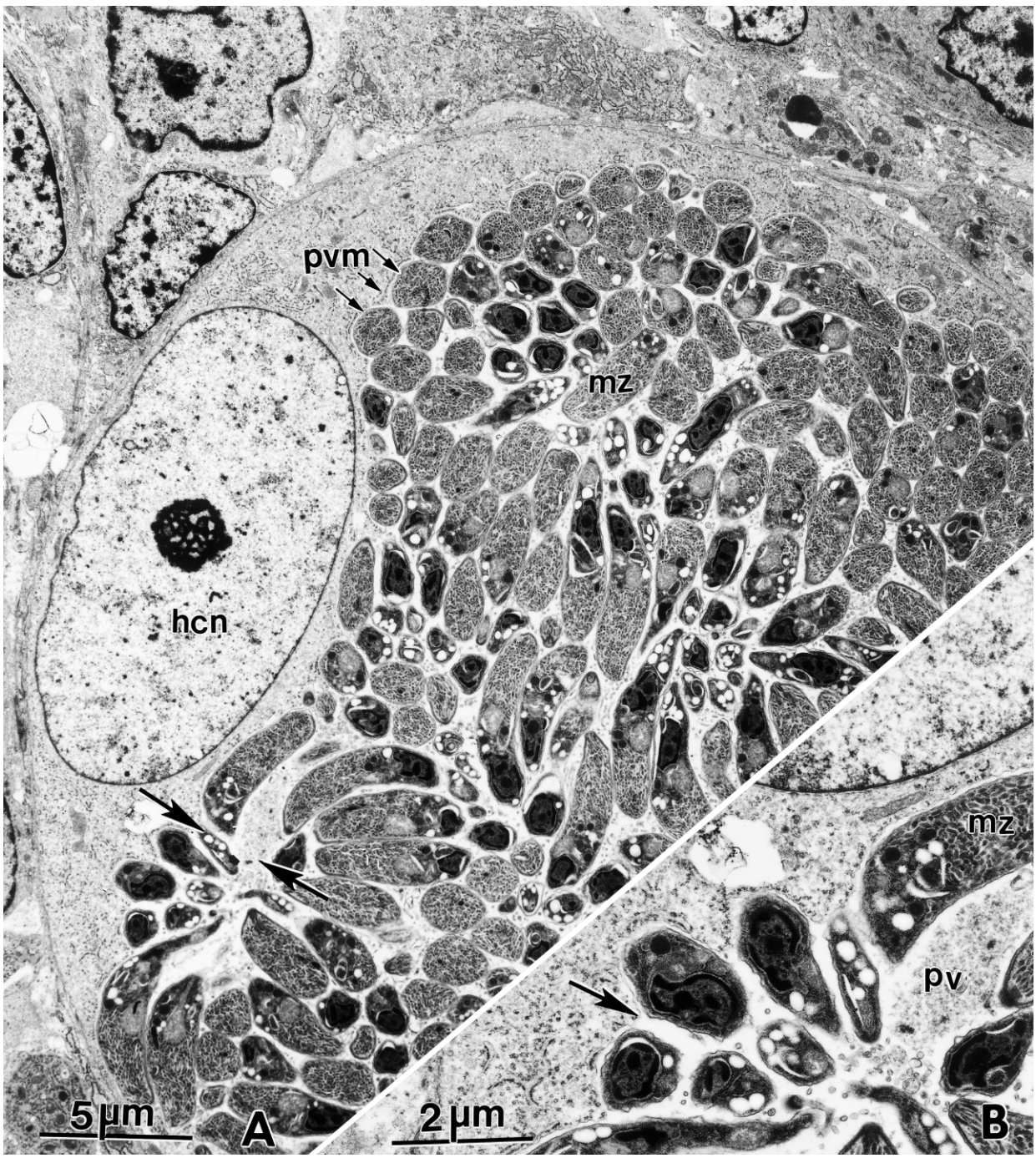

Fig. 9. A mature *Besnoitia oryctofelisi* schizont in the lamina propria of jejunum of the cat. (A) Merozoites (mz) are enclosed in a thin parasitophorous vacuolar membrane (pvm), butted next to the host cell nucleus (hcn). Merozoites (arrows) are arranged in groups in the parasitophorous vacuole (pv); conoidal ends of most merozoites (arrows) in one group facing in one direction. (B) Higher magnification of a group of merozoites indicated by opposing arrows in (A). Note degenerated cellular contents in parasitophorous vacuole (pv).

1984; Njenga et al., 1995), or *Besnoitia* sp. (Paperna and Lainson, 2001), although a critical comparison has not been made. Van Heerden et al. (1993) reported absence of enigmatic bodies in bradyzoites of *B. bennetti*. Enigmatic bodies were first reported by Sénaud (1969) in perinuclear region of *B. jellisoni*. In the present study, enigmatic bodies were found mostly at the non-conoidal end, posterior to the nucleus. Function and origin of these enigmatic bodies are unknown.

4.3. Schizonts and merozoites

The full life cycle of *Besnoitia* is only known for *B. darlingi, B. wallacei*, and *B. oryctofelisi*, and cats are

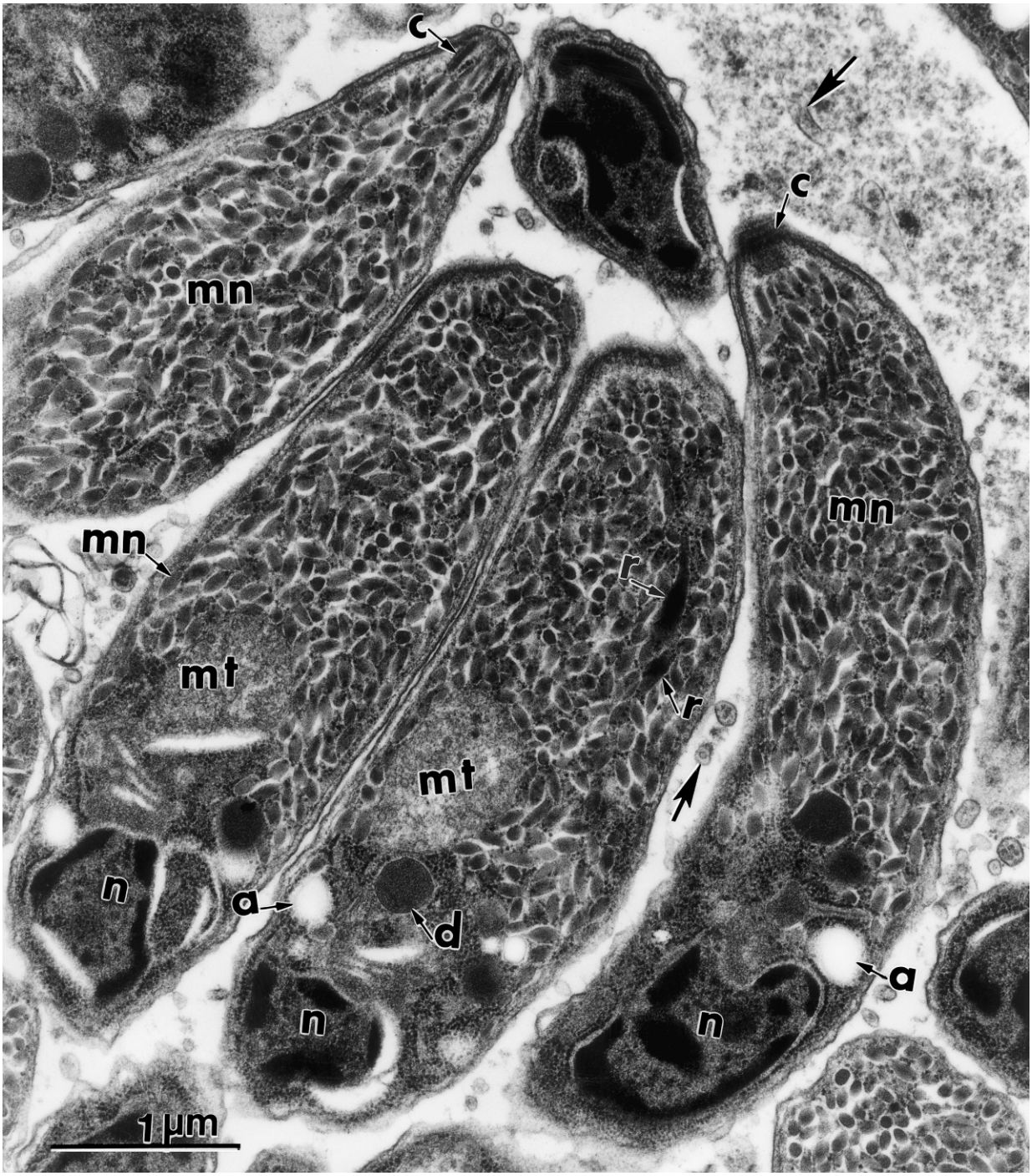

Fig. 10. Higher magnification of four merozoites of *Besnoitia oryctofelisi* indicated by opposing arrows in Fig. 9A. Note numerous micronemes (mn) towards the conoidal (c) ends, two rhoptries (r) in only one of the four longitudinally cut merozoites, a few dense granules (d), a few amylopectin granules (a), a mitochondrion (mt), and a terminal nucleus (n).

their definitive hosts (Wallace and Frenkel, 1975; Frenkel, 1977; Smith and Frenkel, 1977, 1984; Dubey et al., 2002, 2003). Schizonts and sexual stages of these three *Besnoitia* species occur in the intestines of cats. Schizonts of *B. wallacei* are distinct from *B. darlingi* and *B. oryctofelisi* because schizonts of *B. wallacei* are up to 800 µm long and occur in ileum, whereas schizonts of *B. darlingi* and *B. oryctofelisi* are much smaller (<60 µm) in size and occur predominantly in the jejunum (Frenkel, 1977; Dubey et al., 2002, 2003). In size and host location *B. darlingi* schizonts are similar to those of *B. oryctofelisi*. Ultrastructure of only *B. oryctofelisi* schizonts has been studied (present study). Unlike other tissue stages of *Besnoitia*, schizonts develop in intravascularly, and merozoites are produced by schizogony. The schizont nucleus divides into several distinct nuclei before merozoites are formed although

the process has not been confirmed ultrastructurally. Unlike schizonts, *B. oryctofelisi* tachyzoites and bradyzoites divide by endodyogeny.

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